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The genetics of chronic itch: gene expression in the skin of atopic dermatitis and psoriasis patients with severe itch

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Short title: The genetics of chronic itch

Abbreviations: AD (atopic dermatitis), DEGs (differentially expressed genes), FC (fold changes), MRGPR (Mas-related G-protein coupled receptor), Nav (voltage-gated sodium channels), NK-1R (neurokinin-1), PAR (protease-activated receptor), PLA2 (phospholipase A2), PS (psoriasis), RNA-seq (RNA sequencing), TRP (transient receptor potential)
ABSTRACT

To identify itch-related mediators and receptors that are differentially expressed in pruritic skin, we used RNA sequencing to analyze the complete transcriptome in skin from paired itchy, lesional and non-itchy, non-lesional skin biopsies from 25 atopic dermatitis (AD) and 25 psoriasis patients and site-matched biopsies from 30 healthy controls. This analysis identified 18,000 differentially expressed genes common between itchy atopic and psoriatic skin compared to healthy skin. Of those, almost 2,000 genes were differentially expressed between itchy and non-itchy skin in atopic and psoriatic subjects. Overexpression of several genes, such as phospholipase A2 IVD, substance P, Nav1.7, and TRPV1, in itchy skin was positively correlated to itch intensity ratings in both AD and psoriasis. Cytokines such as IL-17A, IL-23A, and IL-31 had elevated gene transcript levels in both itchy atopic and psoriatic skin. However, expression of genes for TRPV2, TRPA1, PAR2, PAR4, and IL-10 was found to be increased only in pruritic atopic skin, while expression of genes for TRPM8, TRPV3, phospholipase C, and IL-36α/γ was elevated only in pruritic psoriatic skin. This “itchscriptome” analysis will lead to an increased understanding of the molecular mechanisms of chronic pruritus and provide targets for itch treatment irrespective of disease state.
INTRODUCTION

The majority of chronic itch patients have a diagnosis of atopic dermatitis (AD) or psoriasis (PS) (Feramisco et al., 2010). Between 87 and 100% of AD patients report chronic itch, making it a defining feature of the disease (Dawn et al., 2009; Yosipovitch et al., 2002). In psoriasis patients, pruritus is the second-most (79%) reported symptom (Yosipovitch et al., 2000). Although pruritus research has advanced in the last decade, the exact mechanisms and genetics involved in AD and PS itch are still unknown. Because itch involves a complex interaction among skin cells, immune cells, secreted factors, and cutaneous neural networks, there is no one specific cause of chronic itch. Furthermore, these components may interact differently in various pruritic disease states and ethnicities (Noda et al., 2015). Identifying the elements that are unique to each disease state and those shared across multiple pruritic diseases is crucial for the development of itch-specific drug therapies.

RNA sequencing (RNA-seq) allows for precise gene identification and quantification of gene expression, even when genes are expressed at low levels (Wang et al., 2009). While a few studies have used genotyping by RNA-seq to examine the genetics involved in AD and PS (Jabbari et al., 2012; Li et al., 2014; Suárez-Fariñas et al., 2015), no study has yet examined gene expression specifically linked to chronic itch. The previous genotypic studies conducted in AD and PS did not include any assessment of itch in their subjects. To better understand itch on the peripheral level, we aimed to assess the genetic expression profiles in the skin of patients with chronic itch from AD and PS, comparing genetic expression with itch severity scores and across ethnicities. After identifying differentially expressed genes of interest, we used immunohistochemistry to examine the level of corresponding protein expression in itchy and
non-itchy skin. This “itchscriptome” analysis elucidates specific genotypic features that define chronic itch in AD and PS.

RESULTS

Analysis of DEGs

This analysis identified 18,000 differentially expressed genes (DEGs) common between itchy, lesional atopic and psoriatic skin compared to healthy skin (Table S1; Tab 1). Of those, almost 2,000 genes were differently expressed between itchy and non-itchy skin in atopic and psoriatic subjects. A heat map summarizing key, established itch-associated DEGs is shown in Figure 1. For these genes, fold changes (FC) and correlations to itch intensity ratings are listed in Table S1 (Tab 2).

DEGs in pruritic skin (compared to non-pruritic skin) in both atopic and psoriatic subjects included those encoding several inflammatory mediators, such as chemokine (C-C motif) ligand (CCL) 2, CCL3, CCL17, CCL18, chemokine (C-X-C motif) ligand (CXCL) 1, CXCL10, chemokine (C-X-C motif) receptor (CXCR) 1, CXCR3, interleukin (IL) 6, IL8, IL17A, IL17F, IL22, IL23, and IL31. TAC1 (substance P), TACR1 (its receptor neurokinin-1 (NK-1R)), transient receptor potential (TRP) VI, Mas-related G-protein coupled receptor (MRGPR) X2, and SCN3A, SCN9A, SCN11A (voltage-gated sodium channels (Nav) 1.3, 1.7, and 1.9) were some of the neuronal components that were overexpressed with the highest FCs. Phospholipase A2 (PLA2) group IV (PLA2G4) B, PLA2G4D, and PLA2G4E, were also prominent DEGs with high FCs that significantly correlated to the itch severity scores. Of note, TPSAB1 (tryptase), HRH2 (histamine receptor 2), kallikrein (KLK) 6 and KLK14, and S100A9 and A15 were also increased in itchy skin.
Several DEGs were unique to pruritic atopic skin or pruritic psoriatic skin. CCL1, CCL2, CCL7, CXCL1, CXCL2, CXCL3, CXCL11, IL4, IL7, IL9, IL10, and IFNG interferon (IFN)-γ were DEGs only in pruritic atopic skin. In contrast, CCL4, CCL7, CCL8, CCL14, CCL20, IL19, IL20, IL26, IL36α, IL36G, and tumor necrosis factor (TNF) α were DEGs only in pruritic psoriatic skin. Interestingly, TRPA1 and TRPV2 had high FCs in the itchy atopic skin, while TRPV3 and TRPM8 were elevated only in itchy psoriatic skin. Other notable genes only overexpressed in itchy AD skin are EDN1 (endothelin-1), EDNRA (its receptor endothelin receptor A), F2RL1 and 3 (protease-activated receptor (PAR) 2 and PAR4), HRH4 (histamine receptor 4), serotonin receptor (HTR) 3B, HTR3C, HTR7, KLK5, KLK13, and S100A2 and G. In itchy PS skin, PLCG1 (phospholipase C γ), MRGPRX3, F2RL2 (PAR3), HRH3 (histamine receptor 3), KLK8, S100A7, and S100P were overexpressed.

**Analysis of DEGs in Ethnic Groups**

Only a few itch-related gene transcripts were significantly different among ethnic groups. TRPA1 was found to be elevated in Caucasians, while African Americans exhibited higher expression of TRPM8 and decreased expression of TRPV1. In the Asian population, S100A2, IL19, and a potassium-dependent sodium/calcium exchanger protein SLC24A5 were overexpressed in itchy skin. The Hispanic population was too limited to reach significant statistical power in this study.

**Immunohistochemistry of Selected DEG Products**

Immunohistochemistry was performed on itchy AD/PS, non-itchy AD/PS, and healthy control skin to validate selected findings from the RNA-seq. TRPV1 and TRPA1 mRNA levels were shown to be increased, but the numbers of TRPV1⁺ and TRPA1⁺ cutaneous nerve fibers were not significantly different between groups. However, the fluorescence intensity for TRPV1
was increased (p<0.0001) throughout the epidermis in itchy AD skin (18.5% increase from non-itchy skin) and itchy PS skin (14.4% increase from non-itchy skin; Figure 2a). TRPA1 fluorescence was also elevated (p<0.0001) in the epidermis of itchy AD skin (34.9% increase from non-itchy skin) but not in itchy PS skin (13.9% increase from non-itchy skin; Figure 2b).

Tryptase and its receptor PAR2 were found to be elevated (p<0.0001) in itchy AD and PS skin. Tryptase was detected in numerous mast cells scattered within the dermis of all skin types. However, most tryptase+ mast cells were located in the papillary dermis at the dermal-epidermal junction in AD and PS skin. Tryptase+ mast cells were significantly increased in itchy AD (40.5.25 ± 6) and itchy PS (42.75 ± 4.5) skin when compared to healthy (6.75 ± 2.25) and non-itchy AD (28.25 ± 9.5) and PS (32.35 ± 4.5) skin (Figure 3a). The largest increases of PAR2 expression were found throughout in the epidermis of itchy AD skin (48.2% increase from non-itchy AD skin). PAR2 was predominantly found in keratinocytes of the granular layer in healthy and PS skin, but there was no significant expression of PAR2 in itchy PS skin (18.4% increase from non-itchy PS skin; Figure 3b).

Immunohistochemical analysis showed that the number of SP+ nerve fibers was increased (p<0.0001) in itchy skin of AD (7.8 ± 1.8) and PS (8.6 ± 2.7) subjects (Figure 4a). This increase of SP was usually seen in nerve fibers that were in close proximity to the dermal-epidermal junction. Moreover, the receptor NK-1R was overexpressed (p<0.0001) within the epidermis of itchy AD skin (88% increase from non-itchy AD skin) and itchy PS skin (30.1% increase from non-itchy PS skin; Figure 4b).

**DISCUSSION**

We have defined an RNA-seq profile for chronic pruritus in atopic dermatitis and psoriasis by comparing differentially expressed genes in pruritic, lesional and non-pruritic, non-
lesional skin. Inflammatory mediators, including many chemokines and cytokines, were commonly overexpressed in itchy atopic and psoriatic skin. With the emergence of new biologic drugs indicated for psoriasis and atopic dermatitis, our data suggest that these treatments should also be tested in other chronic itch conditions. Psoriatic drugs secukinumab and ustekinumab, which target IL-17A and IL-12/23 respectively, may also be effective in atopic dermatitis, where these cytokines are similarly overexpressed (Strober et al, 2016; Kavanaugh et al, 2016).

Likewise, the IL-31 receptor-targeting drug nemolizumab tested in atopic dermatitis should also be examined in other chronic itch diseases (Ruzicka et al, 2017).

The gene transcripts of both the neuropeptide substance P and its receptor NK-1R were elevated in itchy atopic and psoriatic skin. Substance P has been shown to be involved in the mechanism of chronic itch in several disease states, including atopic dermatitis, prurigo nodularis, and psoriasis (Amatya et al, 2011; Järvikallio et al, 2003; Pincelli et al, 1990; Tobin et al, 1992). Accordingly, the NK-1R antagonist aprepitant has been shown to be an effective antipruritic in patients with refractory chronic pruritus from several underlying diseases, such as atopic dermatitis, uremic pruritus, and prurigo nodularis (Ständer et al, 2010). New NK-1R antagonists, serlopitant and tradipitant, are currently being tested in atopic dermatitis and prurigo nodularis. The success of these drugs across disease states may be due to the common overexpression of substance P and NK-1R across multiple chronic itch conditions.

Several itch-associated signaling genes were revealed to have increased expression in itchy skin. Mrgrs, orphan GPCRs, have recently emerged as novel non-histaminergic receptors. MrgprX2 has recently been shown to be activated by substance P to induce inflammation (McNeil et al., 2015). However, this receptor was not antagonized by aprepitant, but by a tripeptide NK-1R antagonist (Azimi et al., 2016), suggesting that antagonists of MrgprX2 may
be of benefit in combination with drug like aprepitant and tradipitant. Recently, Na\textsubscript{v}1.7 was shown to play a role in itch (Lee et al., 2014). While selective Na\textsubscript{v}1.7 antagonists are still in clinical development, an antibody that inhibits Na\textsubscript{v}1.7 did reduce itch in mice (Lee et al., 2014). Also of note, the precursor gene for β-endorphin (\textit{POMC}) was significantly upregulated in both pruritic atopic and psoriatic skin, while the κ-opioid receptor gene (\textit{OPRK1}) was down regulated. This imbalance of μ-opioids may play a significant role in the propagation of chronic itch (Cowan et al, 2015).

Cytosolic members of the group IV PLA2 family of enzymes had significantly higher FCs in itchy atopic and psoriatic skin. These enzymes are involved in cell signaling and the inflammation response via production of arachidonic acid, which is a precursor for eicosanoids. The eicosanoid subfamily of prostaglandins and leukotrienes is known to be involved in itch of AD and PS (Brain et al; 1984; Neisius et al, 2002; Ruzicka et al, 1986). Furthermore, TRPV1, which was also significantly elevated in itchy skin, mediates histamine-induced itching via activation of PLA2 (Shim et al., 2007). Therefore, inhibition of PLA2 may provide a promising antipruritic target.

Of note, \textit{HRH1}, the gene for histamine receptor 1, was not found to be overexpressed in pruritic, lesional skin. H1 antihistamines are frequently given as antipruritic agents, however, these treatments are usually ineffective for chronic pruritus, with the exception of urticaria (Pereira & Ständer, 2017). This supports the theory that most chronic itch conditions are mediated by non-histaminergic pathways (Lavery et al, 2016).

Our study also identified gene transcripts unique to each pruritic disease state. These genes are mainly involved in the inflammatory process or in cell signaling. Our results are consistent with previous RNA-seq studies when comparing lesional to non-lesional atopic or
psoriatic skin. Multiple RNA-seq studies in psoriatic skin revealed enriched DEGs commonly involved in inflammatory response, cytokine-receptor interaction, cell division, and keratinization pathways (Jabbari et al, 2012; Li et al, 2014; Sarkar et al, 2017). These findings include increased expression of several cytokines (IL6, IL12B, IL17A, IL17F, IL21, IL22, IL24, IL26, IFNG, and IFNE), cytokine receptors (IL21R and IL23R), and transcription regulators [signal transducer and activator of transcription (STAT) 1, STAT3, CCAAT/ enhancer binding protein β (CERB), and nuclear factor kappa-light-chain-enhancer of B cells (NFkB1)]. A study in atopic skin found DEGs that consisted of inflammatory mediators [S100A7, S100A8, S100A9, CCL2, CCL3, IL36A, IL36G, IL36RN, triggering receptor expressed on myeloid cells (TREM) 1] and skin barrier proteins [marker of proliferation Ki67 (MKI67) and keratin 16 (KRT16)] (Suárez-Fariñas et al, 2015).

While our genotypic study explored the itch component of atopic dermatitis and psoriasis, we were unable to include analysis of non-itchy, lesional or itchy, non-lesional skin as other controls. Therefore, the observed changes in our itchy, lesional skin might also be a result of scratching. Of note, we only analyzed DEGs in atopic and psoriatic skin that had significant FCs compared to healthy skin. Therefore, while some genes were downregulated in pruritic skin compared to non-pruritic skin, they remained upregulated in non-pruritic AD and PS skin compared to healthy controls. These upregulations in non-pruritic skin may point to underlying differences due to disease state. Additionally, some genes, such as MrgprX1, that have been shown to play a role in itch signaling were not over- or under-expressed in pruritic skin. It is possible that, despite normal mRNA expression, these itch mediators could be dysfunctional in other ways under chronic itch conditions. Our findings among different ethnic groups was limited to genes mainly involved in structural and anatomical differences. Future studies should
focus on phenotyping and genotyping the ethnic differences of chronic itch with larger subject numbers. We were also unable to localize these changes to specific cell types since whole skin biopsies were used in this analysis. However, immunohistochemistry of the selected gene products helped to visualize some of these changes.

This study allowed for the identification of an “itchscriptome” or fingerprint of itch-associated mediators and receptors in atopic dermatitis and psoriasis (Figure 5). Although we found distinct gene expression patterns associated with each group, a common core of components was revealed. The products of these genes are connected to all aspects of itch transmission at the peripheral level and are expressed by skin cells, immune cells, and nerves. While this data does not confirm pruritic mechanisms or drug potential, we believe that treatments targeting these common elements could provide itch relief that is effective across multiple disease states.

MATERIALS & METHODS

Patients

Healthy controls and atopic dermatitis (AD) and psoriasis (PS) patients with severe pruritus (NRS ≥7) were enrolled from Wake Forest University and Temple University in the USA and the National Skin Centre in Singapore (Table 1) in accordance with the Declaration of Helsinki and with approval by each institutional review board. After giving written, informed consent, all subjects provided demographic information, including age, gender, ethnicity, medical history, and current medications. Subjects with chronic itch completed the itch questionnaire and rated their itch in the lesional site with a 0-10 numeric rating scale (NRS). All subjects with chronic itch underwent two 4mm skin punch biopsies, with one biopsy at an itchy, lesional site and the other biopsy at a non-itchy, non-lesional site. One 4mm biopsy,
corresponding to the location of a lesional biopsy, was performed on age- and sex-matched healthy subjects. Half of each biopsy was used for RNA-seq and the other half for immunohistochemistry.

**RNA Isolation and Sequencing**

Skin biopsies were fixed and stabilized using the PAXgene tissue container. RNA was isolated and purified with the PAXgene tissue RNA kit (PreAnalytiX, Switzerland). Purified RNA was quantified with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Nanodrop ND 1000 spectrophotometer (Fisher Scientific, Pittsburg, PA).

Polyadenylated mRNA libraries were prepared using TruSeq Stranded mRNA with Ribo-Zero (Illumina, San Diego, CA). RNA-seq was performed with the Illumina NextSeq (Illumina, San Diego, CA) at a depth of 25 million single-end reads of 75-bp. Quality control was performed on all raw sequence data using FastQC and reads were aligned Using TopHat. Read counts were normalized to the number of reads per kilobase per million mapped reads (RPKM). A Wilcoxon rank-sum test with a Bonferroni corrected p-value (p<0.000001) was used to identify differentially expressed genes (DEGs; based on a false discovery rate less than 0.05 and a fold change (FC) greater than 2.0) in AD and PS pruritic skin versus healthy skin. RPKM data from these DEGs were then analyzed between pruritic and non-pruritic AD and PS skin. These RPKM data (log2 transformed) were also correlated to the itch intensity ratings using Spearman’s correlation analysis. Ethnic influence was analyzed using multivariate logistic regression analysis.

**Immunohistochemistry**

Researchers were blinded to the identity of the biopsies, and the results were only decoded after the immunohistochemical analysis was fully performed. 20-µm thick sections of
paraffin-embedded skin tissue were double-stained from each biopsy. Sections were deparaffinized and then underwent antigen retrieval using Target Retrieval Solutions (DAKO, Glostrup, Denmark) heated in a humidified oven overnight at 60°C, then washed in PBS. Sections were blocked with 5% normal donkey serum and 0.2% Triton X-100 in PBS for 2 hours at room temperature and then incubated with primary antibodies overnight at 4°C.

Primary antibodies were: anti-substance P (SP; 1:1000; ab106291; Abcam, Cambridge, MA); anti-neurokinin 1 receptor (NK-1R; 1:750; PA3-301; Pierce Thermo Scientific, Rockford, IL); anti-tryptase (1:100; ab2378; Abcam, Cambridge, MA); anti-protease-activated receptor 2 (PAR2; 1:100; sc-5597; Santa Cruz, Dallas, TX); anti-transient receptor potential (TRP) vanilloid 1 (TRPV1; 1:200; ab3487; Abcam, Cambridge, MA); anti-TRP ankyrin 1 (TRPA1; 1:500; ab62053; Abcam, Cambridge, MA); and anti-β-tubulin III (1:300; M015013; Neuromics, Edina, MN).

Alexa Fluor (488 & 594, 1:300; Molecular Probes, Eugene, OR) secondary antibodies were used for detection. All slides were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and imaged under a fluorescence microscope. Sections treated without any primary antibodies were used as negative controls. Furthermore, specificity of each primary antibody was confirmed by pre-absorption with its respective peptide in blocking solution overnight at 4°C with gentle agitation. Solutions were centrifuged, and the supernatant was used for immunohistochemistry as described above. In each case, this process resulted in blocking of the primary antibody’s immunoreactivity.

Three fields (20X objective magnification) were measured for every section. The total field and selected field (epidermis) fluorescence areas (in µm²) were measured and normalized to background staining using ImageJ Software (NIH, Bethesda, MD). Data are presented as mean
epidermal fluorescence normalized to mean total field fluorescence. Mast cell (Trytase+ cells) and nerve (β-tubulin III+) counts were also performed using ImageJ Software and normalized to epidermal length as previously described (McArther et al, 1998). All data is reported as mean ± SD or as % change. One-way ANOVAs with Bonferroni post hoc tests were used to compare the differences between groups. Statistical significance was set at p<0.05 (GraphPad Prism, La Jolla, CA).

CONFLICT OF INTEREST
GY has been funded by GSK and the LEO Foundation, and has been an investigator or consultant for OPKO, TREV1, Menlo, Allergen, Eli Lilly, Sanofi Regeneron, Galderma, and Cara.

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# TABLES

Table 1. Subject Demographics and Itch Characteristics

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FIGURE LEGENDS

Figure 1. Heat map of itch-associated gene fold changes (FC) between pruritic, lesional and non-pruritic, non-lesional skin in atopic (AD) and psoriatic (PS) patients. Scale bar represents FC, where blue is underexpressed (FC<2) and yellow is overexpressed (FC >2) in pruritic skin.

Figure 2. The expression of TRPV1 and TRPA1 in atopic, psoriatic, and healthy skin. (a) TRPV1 was overexpressed throughout the epidermis of pruritic atopic and psoriatic skin. (b) TRPA1 was overexpressed in both non-pruritic and pruritic atopic skin. *p<0.05, **p<0.01, ***p<0.001; ****p<0.0001.

Figure 3. The expression of Tryptase+ mast cells and PAR2 in atopic, psoriatic, and healthy skin. (a) A significantly high number of Tryptase+ mast cells were located in the papillary dermis at the dermal-epidermal junction in pruritic AD and PS skin. (b) PAR2 was overexpressed throughout the epidermis in both non-pruritic and pruritic atopic skin. PAR2 was predominantly found in keratinocytes of the granular layer in psoriatic and healthy skin. *p<0.05; ****p<0.0001.

Figure 4. The expression of SP+ nerve fibers and NK-1R in atopic, psoriatic, and healthy skin. (a) SP+ nerves at the dermal epidermal junction were overall increased in AD and PS skin, with the largest increases in the pruritic skin. (b) NK-1R was overexpressed throughout the epidermis in both non-pruritic and pruritic atopic skin, with the highest expression seen in pruritic skin. *p<0.05, **p<0.01, ***p<0.001; ****p<0.0001.
Figure 5. The “itchscriptome” or a fingerprint of itch-associated mediators and receptors in atopic dermatitis and psoriasis. This Venn diagram shows genes differently expressed in both conditions (middle) or unique to atopic dermatitis or psoriasis.

SUPPLEMENTARY MATERIAL

Table S1 Tab 1. DEGs in itchy, lesional atopic and psoriatic skin.

Table S1 Tab 2. Heat map DEG’s